Synopsis

In Silico identification of differentially expressed genes in Triple Negative Breast Cancer and evaluation of anticancer mechanism and modulation of gene expression by Degalactotigonin in MDA-MB-231 Cell line.

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INTRODUCTION

Carcinoma is a complex illness considered by uncontrolled cell growth that is the leading cause of mortality and morbidity universal. Cancer is a multi-step process that begins with a single mutation and progresses over time to generate the first out-of-control cell and, eventually, the tumour. (Eliaa *et al*., 2020). Cancer is the second major consequence of death in the United States, and it is a serious public health problem globally.

Every year, more than 18 million new instances of cancer are diagnosed worldwide. One in every five people will have the condition by the age of 75 worldwide, although this varies from one in ten in Middle Africa and South-Central Asia to one in 2.5 in New Zealand and Australia. Cancer claims the lives of around 10 million people each year. The number of new cancer cases in India is expected to reach 1,324,413. (Cao *et al*., 2021). Despite decades of clinical and scientific research, as well as trials of experimental therapies, cancer remains the leading cause of morbidity and mortality (Richters *et al*., 2020).

One of the main causes of cancer-related morbidity and mortality in women globally is breast cancer. Since breast cancer was first reported by the ancient Egyptians more than 3500 years ago, in or around 1500 B.C., it has a very long history. Breast cancer is currently the second most common type of cancer and is a major factor in the majority of cancer-related deaths in women. (Malvia *et al*., 2017)

Early detection of the disease is critical for efficient treatment and a good prognosis, since individuals with smaller tumors at the time of diagnosis had a reduced risk of death and a greater survival rate. All imaging modalities are consequently focused on early identification of breast cancer and accurate lesion evaluation. Currently, the two key pillars that must be addressed for efficient managing of breast cancer illness are (i) early detection of breast cancer and (ii) rapid therapy after diagnosis to preserve lives. Breast imaging is almost exclusively utilized for cancer detection, diagnosis, and therapeutic treatment, as well as to ensure that breast implants are in good working order. (Iranmakani *et al*., 2021)

Breast cancer treatment requires targeted, effective medicines with little off-target adverse effects. Since this breast cancer is a worldwide issue, a considerable attention should be employed on reducing international inequities in accessibility to screening, multidisciplinary therapy, and innovative medications. Breast cancer is categorised into four molecular subtypes based on receptor expression: oestrogen receptor positive, progesterone receptor positive, and HER2; and lack of these three receptors defines triple-negative breast cancer (TNBC). (Zhang *et al.*, 2018)

Triple Negative Breast Cancer (TNBC) is the most common kind of breast cancer, responsible for 10-15% of all occurrences. TNBCs do not react to hormonal or anti-HER2 treatments because they lack oestrogen and progesterone receptors and exhibit low amounts of HER2. TNBC is a kind of breast cancer that is extremely destructive and has a poor prognosis when compared to other subtypes of breast cancer. (Won and Spruck 2020)

Targeted therapy by combination drug treatment is among the main therapeutic options that can reduce medication side effects while also increasing the success rate of the treatment delivered. It is critical in the case of metastatic cancer patients, as it invades dangerous cells into healthy organs and causes death. As a result, giving the combinatorial drug treatment can assist in improving a synergistic impact with fewer adverse effects (Niemela *et al.,* 2020).

Medicinal plants are being utilized in traditional medicines for thousands of years in many places throughout the world. The empirical information regarding their positive benefits was handed down via human groups throughout the years. Natural products serve an important function as a source of pharmacological compounds, and a number of current medications originating from traditional herbal medicine are presently employed in contemporary pharmacotherapy. (Mukhopadhyay *et al*., 2021)

Plants may generate a wide range of chemical molecules known as phytochemicals, which include both primary and secondary metabolites. Primary metabolites are generally established to contribute to the plant's metabolic processes, whereas secondary metabolites are engaged in a variety of tasks including species interaction, and damage/disease protection.(Xu 2019) Phytochemicals are categorized into three broad classes based on their metabolic origins: phenolic compounds, terpenoids, and nitrogen-containing alkaloids/sulfur-containing compounds. Plants have been utilized as key sources of medicine for millennia. Phytochemicals with therapeutic uses have a wide range of biological actions, including anti-allergic anti-inflammatory, antitumor, antiviral, antimicrobial, and analgesic properties. Similarly, plants have been exploited to create around 25% of the medications used to treat human ailments. (Shin *et al*., 2018)

Solanum nigrum, also known as Maku or black nightshade, is a weed that grows in moist areas with a variety of soils. In conventional medicine it is widely used to treat hepatitis, pain, inflammation, fever and possesses anti-tumorigenic, antioxidant, anti-inflammatory, hepatoprotective, diuretic, and anti - pyretic properties. The plant's juice is used to cure ulcers as well as other skin problems, while the fruit are being used as a laxative, appetite stimulant and

extreme thirst treatments. Several investigations have also shown that the plant suppresses the growth of cervical cancer. Several compounds have been reported as being responsible for various activities. *S. nigrum* is widely used in many traditional systems of medicine around the world for a variety of ailments, but it has received relatively little attention for pharmacotherapeutic use. (Sayeed *et al*., 2021). Degalactotigonin (DGT) is an anticancer compound derived from the *Solanum nigrum* L. plant. It has no major adverse effects. (Mukhopadhyay *et al.,* 2018)

Degalactotigonin, a steroidal glycoside found in this plant, was found to be highly toxic to a variety of cell lines. This is the most anticancer steroidal glycoside isolated from *S.nigrum* so far. It suppressed the growth and spread of osteosarcoma. (Anh *et al*., 2018) Degalactotigonin ($C_{50}H_{82}O_{22}$) from *Solanum nigrum* has the potential to enhance existing pharmaceutical products used to treat various disorders. Many studies have shown that DGT acts as an antifungal agent against the plant pathogen *Pyricularia oryzae*, has cytotoxic activity against several cancers, including human colon, prostate, lung, breast (NCI-H1299, NCI-H460, HepG2, MCF-7, SF-268, NCI-H460, A549, NCI-H1975, and NCI-H1299), and pancreatic cancer cell lines (Zhao *et al*., 2018).

The hypothesis set up for the present study are

Null hypothesis: Degalactotigonin does not possess anti-triple negative breast cancer activity.

Alternative hypothesis: Degalactotigonin possesses anti-triple negative breast cancer activity.

In this study, we proposed to explore the anti-cancer activity of Degalactotigonin in Triple Negative Breast Cancer cell line (MDA MB 231). The objectives framed for the present investigation were

- To compare the gene expression profiles of normal and TNBC patients using *in silico* studies.
- To ascertain the anticancer mechanism of DGT in TNBC cell line (MDA MB 231).
- To explore the molecular mechanism of action of DGT.
- To assess the modulation of gene expression by DGT in TNBC cells.

Based on the advantages of Degalactotigonin, the current study aims to identify a molecular target for TNBC as well as a safe pharmacological approach to minimize chemotherapy side effects. We analyzed TNBC microarray profiles and examined the tumor amplified genes that are prevalent among different TNBC microarray profiles and highly specific to TNBC. We also investigated the synergistic effect of the phytocompound degalactotigonin as well as the

chemotherapeutic drug etoposide in the MDA-MB-231 cell line for anticancer activity and the modulation of selected consistently differentially expressed genes in response to degalactotigonin and etoposide treatment.

To accomplish the above-mentioned objectives, the study was designed to be carried out in four phases.

Phase I

In the first phase of the study, we attempted to identify novel markers of poor prognosis in TNBC and to identify new therapeutic targets for this difficult disease. Bioinformatics methods were used to analyse gene expression profiling data downloaded from the Gene Expression Omnibus (GEO) database to detect differentially expressed proteins (DEGs) between TNBC and healthy human breast tissue. The overlapped differentially expressed genes from three different datasets were scrutinized using the online software Venny 2.1.0. Up and down regulated genes, Gene ontology and pathway analysis was done by Network analyst.

In phase I three gene expression datasets (GSE65194, GSE61724 and GSE38959) were selected for this study. GSE65194 microarray datasets comprising 17 TNBC patients and 11 non-TNBC patients samples were collected from the GPL570 platform. The GSE61724 dataset includes 16 TNBC and 4 Non TNBC patient samples, whereas the GSE38959 dataset includes 30 TNBC and 13 Non TNBC patient samples. Datasets allows researchers to compare the up and down regulating genes in Triple Negative Breast Cancer patients' microarray data to those in non TNBC patients' microarray data.

The Network Analyst platform is a global network platform for assessing genomic expression. Based on the log Fc value, the differentially expressed genes in up and down-regulated genes were determined. The findings indicated that the three datasets comprise 2552 up-regulating genes, with GSE65194 having 2,153 up-regulating genes, GSE61724 having 137 up-regulating genes, and GSE38959 having 262 up-regulating genes. GSE65194 has 21628 down-regulating genes, GSE61724 has 119 down-regulating genes, and GSE38959 has 50 down-regulating genes, totaling 21,797 down genes from three data sets. 64 common up regulating genes and 15 down regulating genes from the data sets GSE65194", "GSE61724" and "GSE38959 were scrutinized using venny software. From the scrutinized up and down-regulating genes, proteins structures were

downloaded using Protein data bank for docking studies. 22 proteins have structures of up-regulated genes whose structures were available and 9 proteins structures of down-regulated genes were retrieved from PDB. Our study investigated the primary modulatory influencing genes of Triple Negative Breast Cancer. The compound degalactotigonin does not attain Lipinski's "rule of five" drug likeness attributes, according to whether a candidate will have less than 500 Da molecular weight, fewer than 5 hydrogen bond donors, fewer than 10 hydrogen - bonding acceptors, and less than 5 partition coefficient (logP) to perform as a people should be treated, rather that the compound degalactotigonin violates the three rule.

The docking techniques showed that the DGT had the best binding affinity to the up-regulated proteins 2RKU, 2Y7B, and 5OJJ, as well as the down-regulated proteins 3U7U, 3KEI, and CCL28, as per the findings of molecular docking studies. The outcome of this finding implies we can focus our research to identify therapeutic compounds to these important modulatory genes.

Phase II:

In this part of the study, we compared the anticancer effectiveness of the compound Degalactotigonin with standard drug Etoposide in the Triple Negative Breast Cancer cell line MDA MB 231 by assessing antiproliferative, antimetastatic, and anticancer activities. We proceeded by treating the Triple Negative Breast cancer cell line MDA-MB-231 with various concentrations of Degalactotigonin at different time intervals to optimize the dose and level of exposure to the steroidal compound. Degalactotigonin was taken in 7 different concentrations ranging from 1 μM to 32 μM at four different time intervals namely 12 hours, 24 hours, 48 hours and 96 hours. . The main aim of the study is to check the efficacy of the compound degalactotigonin compared with the standard drug etoposide in the cell line MDA MB-231. The treatment groups set up were cells alone (control), cells with Degalactotigonin, cells with etoposide and combination of DGT and ET.

The outcome of dose optimization revealed that the IC_{50} value was found to be 8 μM, with a 24 hour exposure time being the optimum. Further research was conducted considering the IC50 value and a 24-hour exposure time interval as the optimal exposure time period. When compared to the other treatment groups, the combination of DGT and etoposide resulted in tremendous loss of cell viability in MDA MB 231 cells.

Degalactotigonin causes cell death via a DNA fragmentation, which is suggestive of apoptosis. Degalactotigonin (IC_{50} = 8 micro molar) and the standard medication etoposide (200 mg) were administered to MDA MB 231 cells for 24 hours before DNA was extracted and separated using agarose gel electrophoresis. The combination of DGT and etoposide showed significant DNA fragmentation, but untreated cells showed minimal DNA fragmentation. These findings demonstrate that the combination of DGT and etoposide successfully promotes apoptosis in MDA MB 231 cells.

Migration is a necessary property of live cells for appropriate development. The cell migration assessment was conducted by scratch assay in MDA-MB-231 cells. The exposure to DGT and etoposide reduced the number of migrating cells significantly, and the group treated with a combination of DGT and etoposide had less number of migratory cells. This suggests that the combined therapy inhibited invasion and metastasis in a synergistic way.

The colony forming assay was used to evaluate the phytocompound's and standard drug's long-term inhibitory impact. When compared to the control group, the number of colonies in the treated group with DGT alone showed a significant reduction. This reveals Degalactotigonin's capacity to effectively suppress colony formation. The number of colonies was also reduced in the group treated with both DGT and etoposide, demonstrating that the combination of DGT and etoposide were effective in inhibiting colony formation.

Propidium iodide and Hoechst staining were done to assess apoptotic cell death and nuclear condensation. As degalactotigonin and etoposide were combined, there was considerable apoptotic cell death and nuclear condensation when compared to the control.

Phase II results, it was clear that the combination of degalactotigonin and etoposide demonstrated improved cytotoxicity, anti-proliferative, and anti-migratory effects, indicating that the percentage of viable cells in triple negative breast cancer cell line was significantly reduced.

Phase III

Phase III was designed to assess the mode of cell death and ascertain the changes involved.

In vitro apoptotic cells eventually return to secondary necrosis. Control, Degalactotigonin, and Etoposide treated TNBC cells were stained with Annexin V-FITC/PI and examined by flow cytometry to evaluate the presence of apoptotic and necrotic cell populations. FITC offers fluorescence for detection when Annexin V attaches to phosphatidyl serine, which is exposed on the exterior surface of the plasma membrane during early stages of apoptosis. Because PI is a membrane impairment nucleic acid stain that is normally rejected by living cells, it was utilized as a counter stain to detect necrotic cells. With MDA MB 231 cells, Degalactotigonin and Etoposide caused a larger percentage of late apoptotic cells than early apoptotic cells. We believe the TNBC cells were experiencing apoptosis based on these findings.

The extent of cell death mediated by apoptosis was observed in MDA MB 231 cells treated with Etoposide and Degalactotigonin, as well as changes in the cell cycle and the distribution index. The untreated control cells were uniformly dispersed in all phases, namely, SubG_0-G_1, G_0-G_1, S, and G_2M, and normal cell cycle events were observed in this group. In the case of degalactotigonin alone treated MDA-MB-231 cells, there was a greater percentage of cells in the G0-G1 phase, whereas etoposide arrested the cell cycle at the S phase and combined treatment arrested the cells at the S and G2/M phases, indicating late apoptotic cell cycle arrest. This shows that degalactotigonin may be utilised successfully to improve cytotoxicity when used in combination with standard drug etoposide to treat triple negative breast cancer cells. Degalactotigonin, Etoposide, and a combination of DGT and ET indicated a change in arrested cells to a Sub-G1 population, also known as apoptotic cells, as compared to control Sub-G1 population growth. The presence of sub-diploid DNA content in a population of cells indicates that DNA fragmentation occurs during apoptosis.

ROS has a critical role in the production of apoptosis in both normal and pathological conditions. The intracellular peroxide level was evaluated by measuring the fluorescence intensity to further establish if ROS generation is involved in apoptosis triggered by Degalactotigonin.

The control group the level of ROS generation was found to be less whereas in the level of ROS significantly increased on exposure to Etoposide and degalactotigonin. The combination of degalactotigonin with etoposide further increased the levels of ROS when compared to control cells and other treatment groups. Exposure to Etoposide alone showed 55% of cell death because

of aggregation, whereas degalactotigonin and degalactotigonin with etoposide caused 57% and 80% respectively.

Thus, the phase III observations further confirmed our previous findings that the compound Degalactotigonin inhibited cancer cell migration. It induced cell death by apoptosis, which was triggered by ROS. In MDA MB 231 cells, cancer cell proliferation was inhibited while apoptosis was elevated, as evidenced by increased DNA damage and cell division stoppage. Our findings that degalactotigonin and etoposide both enhanced DNA damage and apoptosis in MDA-MB-231 cells while inhibiting cell growth.

Phase IV

The impact of Degalactotigonin on the expression of apoptotic genes in Triple Negative Breast Cancer cells (MDA MB 231) was studied in phase IV.

In phase I we identified the differential expressed genes from microarray data sets. To validate that RT-PCR was performed to confirm regulation of up and down genes treated with etoposide and degalactotigonin. The up regulated genes were BUB1, BUB1B, CDC6, and PLK1 mRNA.

Up-regulated genes of TNBC namely BUB1, BUB1B, CDC6, and PLK1 mRNA levels increased after treatment with the chemical Degalactotigonin and the conventional medication etoposide. BUB1 mRNA levels rose considerably in Triple Negative Breast Cancer cells, but did not alter appreciably in etoposide-treated TNBC cells. Degalactotigonin exposure also modified BUB1 expression retrogradely, and DGT and etoposide treatment together generated substantial alterations in triple negative breast cancer cells. BUB1B mRNA expression was higher in the control group than in the etoposide and DGT groups.

Similarly, the DGT and etoposide showed a large fold difference in expression when compared to the control group, showing that BUB1B expression will be elevated in breast cancer. DGT and a combination of DGT and etoposide lowered CDC6 mRNA expression considerably. The combination of DGT and ET improves breast cancer management through modulating CDC6. According to quantitative RT-PCR (qRT-PCR) study of messenger RNA (mRNA) levels, DGT has altered the expression of PLK1 gene when compared to control. Similarly, combining DGT and etoposide resulted in a substantial fold difference, showing that the combination was more effective than either DGT or etoposide alone.

Down-regulated genes of TNBC namely CCL28, NRG1, and PIGR levels increased after treatment with the chemical Degalactotigonin and the conventional medication etoposide. CCL28 appears to be implicated in a number of other cancers. NRG1 is a HER3 receptor ligand that forms heterodimers with other HER family receptors and alters many signaling pathways, including growth, decreased apoptosis, proliferation, angiogenesis, and cellular migration. PIGR has been connected to a number of cancers, including breast cancer. This study screened the mRNA levels of CCL28, NRG1, and PIGR in triple negative breast cancer cell line. The levels of CCL28 mRNA in the DGT and etoposide groups had returned to normal, but had significantly increased in the DGT and etoposide groups. When compared to the other four groups, the control group had the lowest amount of CCL 28. Similar findings were seen in the expression of the genes NRG1 and PIGR after treatment with DGT and Etoposide.

The current study's findings confirmed that degalactotigonin had a substantially differential expression when given along with etoposide demonstrating significant anticancer efficacy. Its efficacy can be exploited in combitorial drug reaction along with standard etoposide therapy can be recommended to reduce the dose of etoposide. Moreover its ability to influence specific genes can be exploited for targeted treatment to treat Triple Negative Breast Cancer cells.

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